CHAPTER 4

Implications of automated Creatine Kinase (CK)-MM1,2,3/CK-MB1,2 isoform analysis as an early marker for the detection of myocardial tissue damage.

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Summary
Measurement of creatine kinase (CK) isoforms enables the clinician to detect myocardial tissue damage in an early stage after myocardial infarction. According to the manufacturer's specifications, it should be possible to perform CK isoform analysis automatically using the new Cardio Rep analyser. In order to investigate the suitability of this new analyser we measured the (CK)MM1-3 and (CK)MB1 and 2 isoform patterns firstly in 30 patients with acute myocardial infarction (AMI) for whom CK-total and CKMB levels were ordered and secondly in 23 patients with chest pain suspected as having AMI (n=11) or with unstable angina pectoris (UAP) (n=12) .
The total time for analysis, including 5 min pre- and 10 min post-analyser run time, was found to be 40 minutes. For elevated MB2/MB1 ratios there is a discrepancy between the MB2/MB1 ratios determined from the densitometric scans concerning the surface and the peak height ratios. The MB2/MB1 ratios of the studied AMI patients exceeded the upper reference limits approximately 2 h after the onset of symptoms, whereas the CK-MB and CK total levels increased after about 6 h. The MB2/MB1 ratios from the patients with UAP were either below the detection limit or these patients could be discriminated from patients with AMI when low CK-MB CKtotal levels were considered in conjunction.
From our results we conclude that assessment of CK isoforms can be performed relatively simply with the new analyser within 40 minutes. However, for reliable calculation of the MB2/MB1 ratios, the curve monitoring of the MB2-MB1 densitometric scans should be improved. The CK isoforms are useful as an early marker for AMI as their reference interval is already exceeded approximately 2 h after an AMI. Moreover, CK isoform analysis might prove to be useful in discriminating at an early stage between AMI and other causes of chest pain. This could decrease the number of - patients with a false-positive diagnosis admitted to Coronary Care Units, resulting in a reduction of costs.

Introduction

Acute Myocardial Infarction (AMI) is an important cause of death in the Western World. Nevertheless, the diagnosis is not always easy to confirm. The recommendation of the World Health Organisation (1) for the diagnosis AMI states that two of the following three criteria should be fulfilled:
(1) typical anamnestic signs (chest pain >20 min, resistance to nitroglycerine);
(2) typical ECG findings (serial ECG >24 h: ST elevation 1.5 mV in precordial leads or/and ST elevation >1.0 mV in standard leads followed by T-wave inversion);
(3) increase in the serum cardiac enzymes lactate dehydrogenase (LD, EC 1.1.1.27), aspartate aminotransferase (ASAT, EC 2.6.1.1) and creatine kinase (CK, EC 2.7.3.1).
Several criteria have been proposed for an 'ideal' marker for detection of AMI (2). It should only be present in high concentration in myocardial tissue. After an AMI the marker should be released rapidly and its level should be proportional to the damage of myocardial tissue. The period following an AMI during which the marker can be demonstrated in the blood should be long enough for detection. On the other hand, the period should not be too long, so that reinfarctions might be overlooked. To date, no commercially available fulfils all these criteria.
For more than 20 years the enzyme activities of CK total and CK-MB have been used for the early detection of AMI. Early detection is important, since the prognosis of patients with AMI is

considerably improved, if therapy to restore the coronary blood flow is started within 6 h of the onset of symptoms (3-5). Early detection of myocardial damage depends on the appearance in the blood of increased levels of markers of myocardial tissue damage. The latter increase depends partly on the molecular mass of the marker. Therefore, the determination of smaller proteins in serum or in plasma is a reliable tool for the early diagnosis of AMI. Myoglobin (6,7) and heart fatty acid binding protein (8,9) are examples of such small muscle proteins. Troponin T (10-13) and Troponin I (14-17) are alternative markers. Recently glycogen isophosphorylase BB (18) and CK isoforms (19-22) have been reported as ideal markers for the early detection of AMI.

Creatine kinase consists of the isoenzymes CK-MM, CK-MB and CK-BB (23). CK-MM has three subforms (24,25): (CK-)MM1 (plasma), (CK-)MM2 (intermediate plasma) and (CK-)MM3 (tissue). The conversion of MM3 into MM2 and of MM2 into MM1 by cleaving the C-terminal amino acid lysine of each CK-M-subunit is catalyzed by plasma carboxypeptidase (26). For CK-MB only 2 subforms (27) are detectable in serum: (CK-)MB1 (plasma) and (CK-)MB2 (tissue). The conversion of MB2 into MB1 is also catalysed by plasma carboxypeptidase. MB1 is inactivated by proteolysis in the lymph (28).

Puleo et al. have shown that at 6 h after an AMI plasma CK isoforms have a diagnostic sensitivity and specificity of 92% and 96% (29), respectively. In the latter study the Rep analyser (Helena Laboratories, Beaumont, USA) was used to determine the levels of the CK isoforms. Analysis with this instrument is labour-intensive and time-consuming because of the numerous manual actions involved in loading of the sample, electrophoresis, detection and scanning of the isoform bands. Thus this procedure is not suitable for CK isoform analysis at any time, as needed. The new Cardio Rep has overcome this drawback.

We investigated the performance of the new analyser and the changes in CK-MM and CK-MB isoform patterns as a marker for the detection of myocardial tissue damage in patients in whom there was a high suspicion of AMI. In addition, we discuss the possible implications, for the patient and the hospital organisation, if the automated CK-isoform determination is introduced into everyday clinical practice.

Materials and Methods

Patients

To investigate the performance of the new analyser and to find out whether it is user-friendly, CK isoforms were measured from 30 patients with AMI for whom determinations of CK total and CKMB-activities were ordered.

Also 23 patients were studied who were admitted to our hospital with chest complaints, where there was a suspicion of unstable angina pectoris (UAP) (n=12) or AMI (n=11). All patients were treated with intravenous nitroglycerin, heparin, and oral aspirin. Out of these 23 patients, 11 patients (seven male, mean age 60 years, median 61; four female, mean age 64 years, median 62) had had an AMI and 12 patients (six male, mean age 63 years, median 62; six female, mean age 74 years, median 72) had experienced UAP.

The patient materials used for electrophoresis were serum samples. Serum was separated from the blood cells after the blood was centrifuged at 1000x g during 10 minutes. The analyses were performed within 24 h after blood sampling. Although the manufacturer recommends EGTA plasma samples, we found no significant difference between results obtained from EGTA plasma samples and those obtained from serum samples.

Methods

The MM1-MM3 and MB1 and MB2 isoform patterns were determined using the Cardio Rep. Before the analyser run can be started, it takes 5 min to apply the thin-layer 12 g./l agarose gel, to put the sera samples into the wells and to enter the sample identification.

After the analyser run has been started "sample applications" appears on the screen. During this procedure, lasting 3 min, excess buffer is removed, the applicators are washed and the samples are applied and absorbed in the gel.

The next procedure is electrophoresis at 25 °C, 900 V, 40 mA for 6 min. The gel is cooled by a thermostatically controlled peltier support to prevent melting of the agarose and denaturation of the CK enzyme.

Electrophoresis is followed by reagent application procedure. During this procedure, which takes 3 min, the standard reagent for CK analysis (30) is poured, spread and absorbed, and finally the excess of reagent is removed. The next step is incubation for 5 min at 50 °C. After incubation the gel is dried at 55 °C for 4 min.

Finally, the scan procedure begins with cooling of the gel to 20 °C, followed by determination of the optimum PMT voltage (approximately 400 V), scanning (wavelength 340 nm) of the MB1 and MB2 bands of the samples, editing and saving the scans. The scan procedure takes 4 min. The entire standard analyser run, which measures only the MB2/MB1 ratio, takes 25 min.

After the standard analyser run has finished, there is another 10-min procedure for additional scanning, editing and saving of the MM1-MM3 bands, the calculation of MM3/MM1 ratios, and inspection and printing of the Mb and the MM scans.

Thus, the total analysis time takes 40 min, and consists of 25 min for the actual analyser run time, plus 5 min before and 10 min after. Five patient samples can be analysed simultaneously with one standard sample on one gel in the same run.

The levels of CK total (reference values men < 70 U/l and women <50 U/l) and CK-MB (immune-inhibition) (reference values < 10 U/l) were determined using an Ektachem analyser (Johnson and Johnson, Beerse, Belgium).

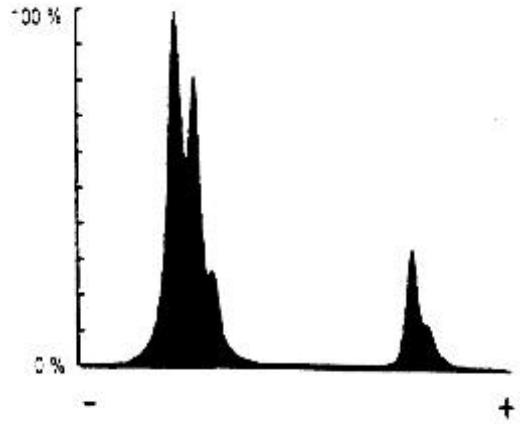

Figure 1. A typical isoform pattern after electrophoresis and scanning of the creatine kinase MM1, MM2, and MM3, and MB1and MB2 isoforms in a patient several hours after myocardial infarction. The cathode is represented by - and the anode by +. The most intense isoform band is defined as 100%.

Results

A typical pattern of the densitometric scan after CK isoforms analysis from a patient with AMI is shown in figure 1: between the cathode and the anode the MM3, MM2, MM1 and the MB2, MB1 peaks are shown. As already described in the Patients and methods section, the total running time of the determination is about 40 min.

In Figure 2A the MB2/MB1 ratios obtained from 30 AMI patients are plotted calculated from the MB2/MB1 surface ratios and from the MB2/MB1 peak height ratios. The surface ratio was calculated by the analyser from the densitometric scan.

A **B**

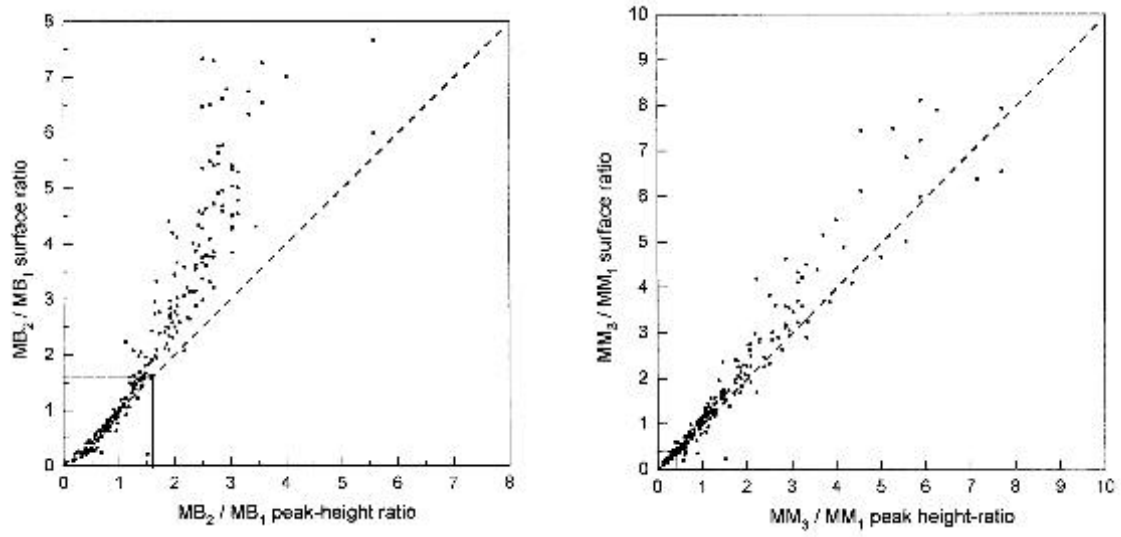

*Figure 2.*The relations between (A) the MB2/MB1surface ratios and the MB2/MB1 peak height ratios, and (B) between the MM3/MM1 surface ratios and the MM3/MM1 peak height ratios. The surface ratios are generated from the scans after electrophoresis by the standard software of the analyser. The peak height ratios are calculated manually from the scans visualised on the monitor of the analyser. For the MB2/MB1 ratio the cut off value line is drawn at 1.6 and for the MM3/MM1 ratio at 0.4. The results are derived from 30 patients with acute myocardial infarction (AMI).

The peak height ratio was manually determined from the densitometric scan visualised on the screen of the monitor. It can be seen from Figure 2A that up to the cut-off value of 1.6 there is no discrepancy between these differently calculated MB2/MB1 ratios. Above the 1.6 cut-off value the surface ratios are higher than the peak height ratios. The MM3/MM1 surface and peak height ratios are plotted in Figure 2B, showing that there is a good correlation between the MM3/MM1 surface and peak height ratios.

The MB2/MB1 surface ratios have also been measured in 23 patients who were hospitalised for typical chest pain and ECG changes. In Figure 3 the MB2/MB1 surface ratios and the CK-MB levels (still routinely determined in our hospital) plotted against the time after infarction for the first 24 hours after the infarction, are represented for 11 patients with AMI. The MB2/MB1 ratios are above the cut-off value of 1.6 (12) at approximately 2 h after the infarction. At that time the CK total and the CK-MB levels are still within reference ranges for AMI as well as UAP patients. At admission to the hospital the CK total and the CK-MB levels of the UAP patients (mean activities 45, 3 U/l respectively) are comparable with those of the AMI patients (60, 4 U/l, respectively). The CK-MB activities are 'positive' (i.e. above the upper limit of the reference interval) approximately 6 h after the onset of the anginal complaints. At that time the MB2/MB1 ratios have maximal values,

whereas the CK-MB activities reach these maximal values at about 15 h after the onset of the anginal complaints.
Out of eleven patients with AMI, eight had maximal MB2/MB1 surface ratios between 4 and 6, one had a ratio lower than 4 (i.e. 2.6) and two had ratios higher than 8 (i.e. 8.1, 9.3). The MB2/MB1 ratios for UAP patients are lower. For seven out of 12 patients with UAP it proved impossible to determine the MB2/MB1 ratios, because the MB2 and MB1 levels were below the detection level. For five patients the maximal MB2/MB1 ratios were 1.1, 1.7, 1.8, 1.9, 2.9 respectively. None of these patients had maximal CK activities above 100 U/l or maximal CKMB activities above the upper limit of reference interval.

Discussion

CK isoform analysis with the new Cardio Rep analyser is relatively simple as a result of the automated electrophoresis procedure. The total running time of the analysis is approximately 40 min consisting of 5, 25 and 10 min pre-run, actual run and post-run time. This is comparable to the assay time of most of the other cardiac markers. In these experiments the analyser proved to be user-friendly and easy to operate.
However, the analyser would become more user-friendly, and the post-run time can be shortened by some minutes, if the MM1-MM3 bands were scanned, edited and saved together with the MB1 and MB2 bands in the same standard program.
Furthermore, the CK isoform assessment would become more reliable, if the curve monitoring of

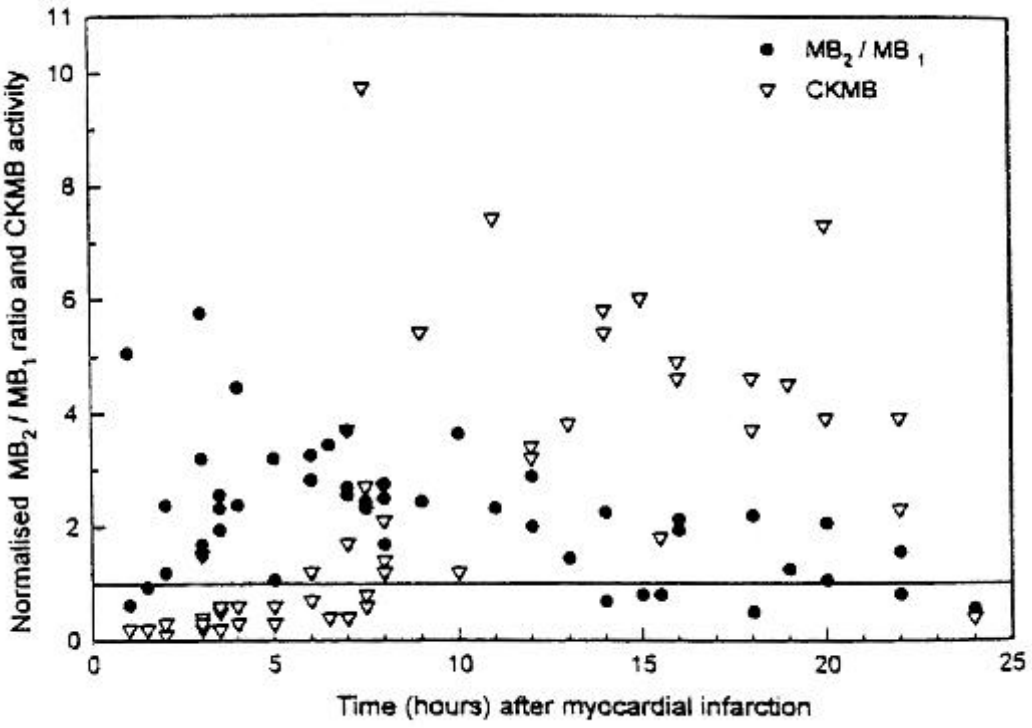

Figure 3. Normalised MB2/MB1 ratios (!) and normalised CKMB levels (L) versus the time after infarction, for 11 patients with AMI for the first 24 h after infarction. The normalised MB2/MB1 ratios and the normalised CKMB levels are multiples related to the cut off values of the MB2/MB1 ratio (1.6) and CKMB level (10 U/L).

the MB1 and MB2 peaks was improved. From the data presented in Figure 2 it is concluded that the curve monitoring of the CK isoforms must be improved in order to measure the MB2/MB1 isoforms ratios more accurately. It is difficult to determine the MB2/MB1 ratio accurately especially when the CK levels are elevated and the densitometric curves partly overlap each other,.

The discrepancy between the MB2/MB1 surface ratios and the MB2/MB1 peak height ratios is caused by the insufficient separation of the isoform bands by the densitometer. The separation of the MB1 and MB2 bands can be enhanced either by improving the electrophoretic separation or by improvement of the curve monitoring software of the densitometer. As increasing the time of electrophoresis requires more gel, this is not a possible solution for the problem of the overlapping MB1 and MB2 bands because of the volume available for the gel in the analyser. Thus, the only solution is to improve the curve monitoring software of the densitometer. With the present analyser software the MB2/MB1 ratios can only be used for the qualitative detection of myocardial tissue damage.

If the MB2/MB1 ratio is above 1.6, we recommend the additional use of the MM3/MM1 ratio. - The MM3/MM1 ratios are more reliable, because the MM3 and MM1 bands cannot overlap each other as they are separated by the MM2 fraction. Moreover, we have found that this ratio is a more reliable indicator of the time after infarction in a single blood sample. The MM3/MM1 ratio cannot be used as a primary criterion for myocardial tissue damage, because skeletal muscle accounts for 97% of MM. For this reason MM has poorer clinical specificity for myocardial tissue damage compared with the MB isoforms.

From our comparison of CK isoforms with CK-MB levels, we conclude that the MB2/MB1 isoform ratios show an earlier increase after AMI than the CK-MB levels still routinely used in our hospital. To provide a reliable diagnosis after an AMI the CK-MB level should show a manifold increase to exceed the reference interval. However, if the diagnosis is based on a change in the ratio of the MB isoforms, a release of small amounts of the MB2 isoform into the plasma leads to a significant change in the MB2/MB1 ratio.

Recently, equivalent early sensitivities of myoglobin, creatine kinase MB mass, creatine kinase isoform ratios and cardiac troponins T and I have been reported for AMI (31). In this study Mair et al. Used the Rep for the measurement of CK isoforms. They reported an increase in the MB2/MB1 ratio 3 h after infarction. In our study we found this time to be approximately 2 h. This might possibly be explained by modifications to the new analyser. As a result of these modifications, the gel is no longer exposed to daylight during anaslysis so that weaker MB1 and MB2 bands can be detected.

There are two essential differences between the information from the CK isoform analysis and that from the other commercially available cardiac markers. First, the CK isoform analysis reports ratios, giving relative rather than absolute results. Second, only the CK isoform measurement offers the possibility of indicating the time between infarction and blood sampling in a single blood sample with a single measurement.

From our experience, we suggest the following use of the Cardio Rep. Usually, patients are diagnosed on the basis of symptoms and ECG changes; laboratory test results are more frequently used for confirmation and quantification of the AMI. The CK isoform analysis can be used in those patients where the diagnosis is doubtful, since the MB2/MB1 ratio is already raised 2 h after infarction. As this technique indicates the time since infarction using a single analysis in a single blood sample, another reason for CK isoform analysis arises when it is not clear how much time has passed since the start of the episode.

The introduction of routine automated CK isoform analysis will increase laboratory costs. On the other hand, a 1-day patient stay in the CCU is much more expensive than the measurement of CK-isoforms. The savings as a consequence of more efficient patient treatment are impressive, considering that in the USA the annual costs of caring for 'non-myocardial infarction' in CCU's are $6 -$13 billion (32,33).
Further studies should be carried out to determine the clinical relevance, for differentiation between AMI and UAP patients of the changes over time in the CK-MB and CK-MM isoform ratios, and to determine the cost-benefit consequences. in time for differentiation between AMI- and UAP-patients and to determine the consequences for the cost- benefit relation. The kinetics of CK isoform alterations (i.e. maximal value of the MB2/MB1 ratios, and the time at which these values are reached) may indicate the time required for patient recovery and patient stay in CCU's. To date, it is not clear whether there are associations between CK isoform kinetics, infarct size and the prognosis for the patient.
In conclusion, with regard to the automated CK isoform analysis using the Cardio Rep, the following is noted: it takes about 40 min in total, including 5 min pre- and 10 min post-analyser run time to automatically measure the CK MM1-MM3 and MB1 and MB2 isoforms, and the analysis is relatively simple; however, the curve monitoring of the MB2-MB1 scan should be improved for reliable isoform ratio calculations; the CK isoforms have the potential to discriminate in an early stage between AMI and other causes of chest pain.
As a consequence of the introduction of CK isoform analysis in daily practice, patients with an AMI can be diagnosed earlier. Moreover, the number of patients admitted to CCU's with a false-positive diagnosis may decrease, resulting in a reduction of costs.

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